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Modification of human haemoglobin with glucose 6-phosphate enhances tetramer–dimer subunit dissociation

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Studies using equilibrium gel-permeation chromatography demonstrate that formation of the covalent adduct of D-glucose 6-phosphate (G6P) with human haemogobin promotes dissociation of the haemoglobin tetramer into its component $\alpha\beta$ dimer pairs [$K_d^{oxy} = 2.57 \times 10^{-6}$ versus K_d^{oxy} (G6P) $= 11.22 \times 10^{-6}$ M-haem]. On the other hand, K_d for glucosylated haemoglobin is identical with those of the O_2- and CO-liganded forms of intact haemoglobin A_0. These data are consistent with the phosphate moiety alone being responsible for a 4.5-fold increase in the tetramer-to-dimer apparent K_d. This suggests the glucose 6-phosphate moiety does not bind to the same sites on haemoglobin as do the free organic phosphates, as suggested by ligand-binding kinetics data or structural analysis. My study presents a working model for studying changes in protein subunit assembly as altered by protein phosphorylations.

INTRODUCTION

Exploring the structure–energy coupling of human haemoglobin and its role in biological function has been approached by perturbing the molecule through alteration of individual specific amino acid residues while observing the altered energetics of ligand binding and/or subunit assembly [1]. Inducing molecular changes by interaction of haemoglobin with specific 'allosteric effector' salts (e.g. 2,3-bisphosphoglycerate) [2] and/or by post-translational modifications (e.g. glucosylations) [3] is also a recognized strategy for exploring this structure–energy coupling. Post-translational modifications are of interest because of their wide occurrence in biological systems and their recognized importance in regulation of protein function (protein phosphorylation etc.) [4].

Studies suggest that the covalent attachment of D-glucose 6-phosphate to human haemoglobin may be functionally analogous to the binding of 2,3-bisphosphoglycerate [3,5]. That hypothesis would suggest that glucose 6-phosphate should stabilize the deoxy or tetramer form of the dimer–tetramer subunit assembly of haemoglobin [6,7,8]. Direct measurement of the dimer–tetramer equilibria of glucophosphorylated haemoglobin would establish a basis on which to support or reject the hypothesis proposing homology between the interactions of 2,3-bisphosphoglycerate or glucose 6-phosphate with human haemoglobin.

In this study I used equilibrium gel-permeation chromatography [9,10] to measure the dimer–tetramer subunit equilibrium constants of human haemoglobins A_0, A_{1c} and A_{G6P}, in both the O_2- and the CO-liganded states. My findings show that glucophosphorylation of human haemoglobin stabilizes the dimer species of this equilibrium. My results are contrary to those expected on the basis of the above hypothesis, and also suggest that glucophosphorylated haemoglobin may be a useful model for studying protein phosphorylation and its effects on protein subunit assembly.

EXPERIMENTAL

Preparation of haemoglobins A_0, A_{1c} and A_{G6P}

Haemoglobin A_0 was purified from freshly drawn heparinized blood by chromatography on Whatman CM-52 CM-cellulose. Haemoglobin A_{1c} (glucosylated haemoglobin) was collected from fractions obtained during the purification of haemoglobin A_0 and subsequently rechromatographed on Bio-Rex 70 (Bio-Rad Laboratories) for purification by the method of McDonald *et al.* [11]. All purification procedures were performed at 4 °C with haemoglobin in the oxygenated form except for the preparation *in vitro* of haemoglobin A_{G6P} (glucophosphorylated haemoglobin). Haemoglobin A_{G6P} was prepared by the method of Haney & Bunn [3] with slight modifications. I used hydrated N_2 gas to deoxygenate the reaction mixture of haemoglobin A_0 with D-glucose 6-phosphate (Sigma Chemical Co.) at a haemoglobin/glucose 6-phosphate molar ratio of 1:10 during a 16 h incubation at 4 °C with constant gentle stirring. The haemoglobins described above were concentrated by using ultrafiltration cells with PM-10 membranes (Amicon). Homogeneity of the various haemoglobins was verified during and after the purification procedures by h.p.l.c. on Bio-Rex 70 [12] (see Fig. 1) and by polyacrylamide-slab-gel isoelectric focusing [13,14] (results not shown). All isolated haemoglobins were stored in liquid N_2 until use.

Measurement of dimer–tetramer equilibrium

Large-zone (plateau) gel-permeation chromatography experiments were performed as described elsewhere [15]. Briefly, Sephadex G-100 (Pharmacia) preswollen in Tris/HCl buffer (0.1 M-Tris/HCl, pH 7.4, containing 0.1 M-NaCl and 1 mM-Na_2EDTA) were the solid and liquid phases respectively. Experiments were done with 1.0 cm × 30.0 cm jacketed glass columns maintained at a temperature of 21 °C. Column flow rates were controlled at 12.0 ml/h by an LKB model 10200 peristaltic Perpex

Abbreviation used in Table and Figures: Hb, haemoglobin.

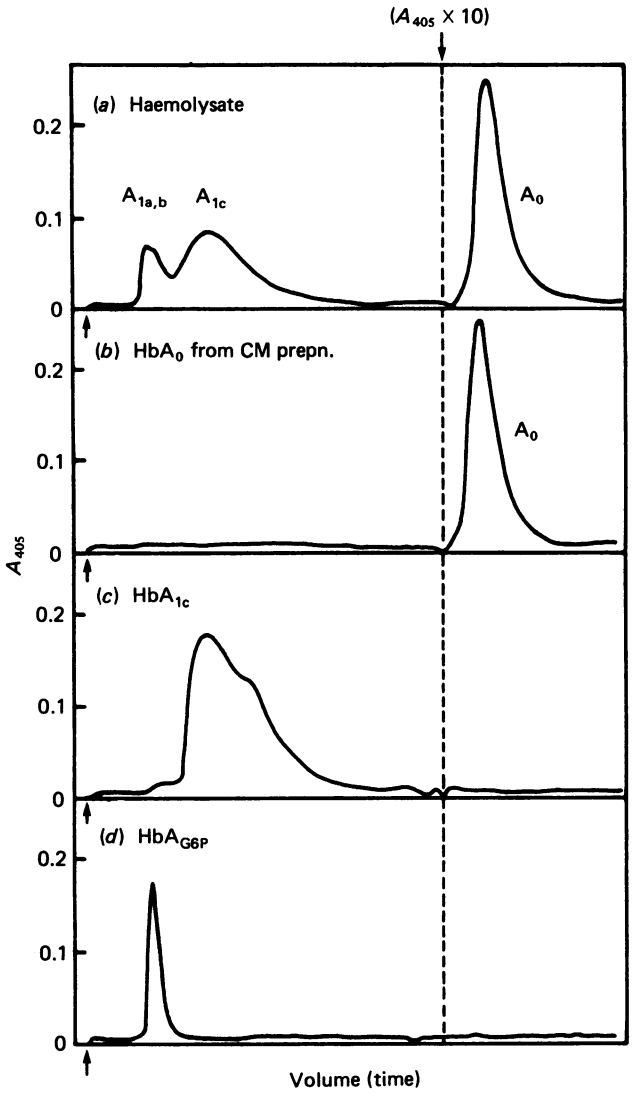

Fig. 1. (*a*) Bio-Rex 70 h.p.l.c. elution pattern of normal haemolysate, (*b*) haemoglobin A_0 purified by preparative CM-cellulose chromatography and run on a Bio-Rex h.p.l.c. column, (*c*) isolates of haemoglobin A_{1c} from the Bio-Rex preparative column run on Bio-Rex 70 h.p.l.c., and (*d*) haemoglobin A_{G6P} product of haemoglobin A_0/glucose 6-phosphate reaction mixture after one passage through preparative Bio-Rex 70 chromatography rechromatographed on a Bio-Rex 70 h.p.l.c. column

pump connected in series subsequent to the spectrophotometric flow cell. Column effluent was monitored by a Beckman DU/Gilford spectrophotometer interfaced with a Fisher Record-All series 5000 strip chart recorder. Throughout these experiments protein concentrations were monitored at wavelengths ranging from 230 to 600 nm as required. Haemoglobin concentrations were determined by using a molar absorption coefficient of 15.4 $mM^{-1} \cdot cm^{-1}$ at 576 nm, based upon pyridine haemochromogen determination. All haemoglobin samples were dialysed by passage through Sephadex G-25 (Pharmacia) columns or by diafiltration cells (Amicon) with the working Tris/HCl buffer described above before being loaded on to the analytical gel-permeation column. Spectrophotometric scans (Varian DMS 90) were used to verify the degree of methaemoglobin formation to be less than 3% by the method of Benesch *et al.* [16] and the states of O_2 and CO haemoglobin saturation before and subsequent to the analytical chromatographic experiments. Calibration of the column was performed with small-zone experiments with known protein markers [15]. Throughout this study several different analytical and preparative columns were used, as well as several haemoglobin preparations, all giving comparable results.

Data reduction

The weight-average elution volumes, V_E, from the large-zone experiments were determined from the centroid position of the leading boundary defined as:

$$V_E = \frac{1}{C_T}\int_0^{C_T} V \cdot dC$$

where C_T represents the plateau contentration, C the total protein concentration within the boundary, and V the elution volume [17]. Data reduction of these subunit dissociation curves (V_E versus C_T) and determination of the apparent dimer–tetramer dissociation constants, K_d, for the reaction:

$$(\alpha_2\beta_2) \rightleftharpoons 2(\alpha\beta)$$

i.e.

$$K_d = (\alpha\beta)(\alpha\beta)/(\alpha_2\beta_2)$$

were performed by non-linear least-squares fitting routines [18] using the relationship:

$$V_E = \Sigma j V_j(m_j)/\Sigma j(m_j)$$

where V_j are the individual species elution volumes pertaining to the various aggregates (j-mers), and the (m_j) terms represent the respective species molar concentrations within the plateau region of total molar concentration, $C_T = \Sigma j(m_j)$. These programs were kindly provided by Dr. M. L. Johnson (University of Virginia) and run on a VAX computer at our institution.

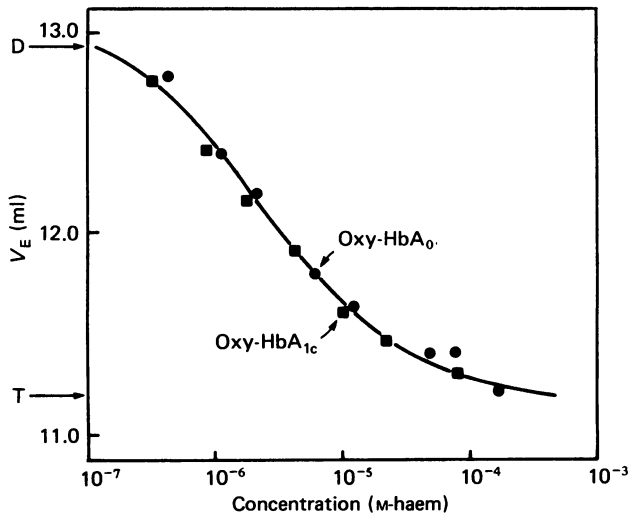

Fig. 2. Sephadex G-100 large-zone elution volumes, V_E, plotted versus total protein concentration, C_T, for oxyhaemoglobin A_{1c} and oxyhaemoglobin A_0 as indicated

Buffer conditions were 0.1 M-Tris/HCl, pH 7.40, 0.1 M-NaCl, 1 mM-Na_2EDTA. Temperature was 21 °C and column flow rate was 12 ml/h. Dimer and tetramer end points were determined as indicated elsewhere [15].

Table 1. Apparent tetramer-to-dimer dissociation constants for glucosylated and glucophosphorylated human haemoglobin

Solvent conditions: 0.1 M-Tris/HCl, pH 7.40, 0.1 M-NaCl, 1.0 mM-Na_2EDTA, 21 °C. One standard deviation to the best fit is shown.

Haemoglobin species	K_d (oxy) (10^{-6} M-haem)	K_d (CO) (10^{-6} M-haem)
HbA_0	2.57±0.21	1.05±0.20
HbA_{1c}	2.60±0.25	1.06±0.14
HbA_{G6P}	11.22±0.18	11.20±0.25

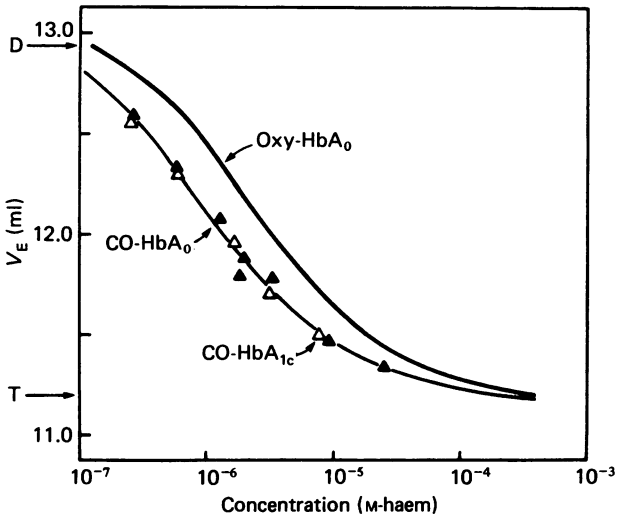

Fig. 3. Sephadex G-100 large-zone elution data for carbonmonoxyhaemoglobin A_{1c} and carbonmonoxyhaemoglobin A_0 as indicated, compared with the elution curve of oxyhaemoglobin A_0 (taken from data of Fig. 2)

Conditions were as given in Fig. 2 legend.

RESULTS

The results of large-zone gel-permeation experiments comparing the apparent tetramer–dimer dissociation constant of fully oxygenated phosphate-stripped haemoglobin A_0 with glucosylated haemoglobin A_{1c} are shown in Fig. 2. The apparent $\alpha\beta$ dimer dissociation constants (see Table 1) for these two species are identical within the precision of my measurements. That is, the presence of the sugar group at the *N*-terminus of the β chains does not alter this subunit equilibrium. Fig. 3 shows the results of the same experiment with the two haemoglobin species A_0 and A_{1c} in the CO-saturated forms. The carbonmonoxyhaemoglobin A_0 shows a lower dissociation constant compared with oxyhaemoglobin A_0, as previously established [19]. This same Figure shows the CO-liganded form of haemoglobin A_{1c} sharing a superimposable subunit dissociation curve when compared with the native non-glucosylated carbonmonoxyhaemoglobin A_0. The results in Figs. 2 and 3 demonstrate the independence of $\alpha\beta$ dimer subunit interaction to the presence of the glucose molecules at the *N*-terminus of the β chains [3]. On the other hand, the results in Fig. 4

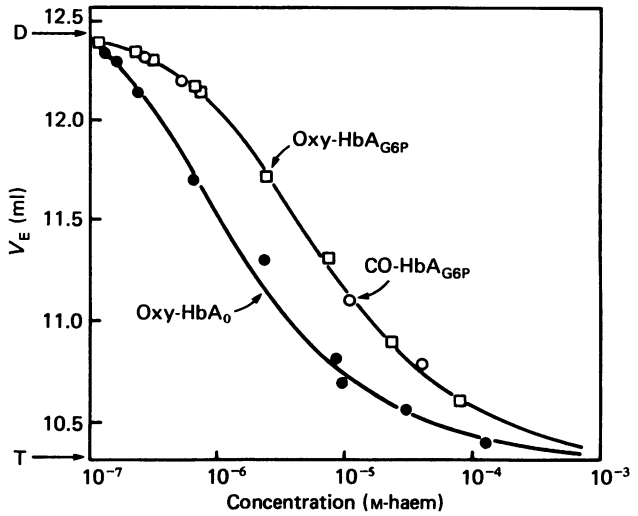

Fig. 4. Sephadex G-100 large-zone elution data for oxyhaemoglobin A_{G6P} and carbonmonoxyhaemoglobin A_{G6P} as indicated, compared with oxyhaemoglobin A_0

Conditions were as given in Fig. 2 legend.

clearly show the profound change in the dimer–tetramer equilibria for the haemoglobin A_{G6P} species compared with haemoglobin A_0. The O_2- and CO-saturated forms of glucose 6-phosphate-modified haemoglobin have identical dissociation curves and therefore apparent calculated constants (Table 1).

DISCUSSION

Three conclusions may be drawn from the results of this present study. The first is that a glucose covalent adduct at the *N*-terminus of the β chain does not alter the dimer–tetramer subunit equilibrium distribution for either the O_2- or the CO-liganded states of human haemoglobin A_0. This suggests the glucose moiety at the *N*-terminus alone does not measurably perturb the structure/function of human haemoglobin. These results are consistent with the findings of other studies demonstrating only minor changes in the O_2-binding properties of glucosylated haemoglobin A_{1c} [20,21]. A second conclusion is that the presence of a phosphate group on *N*-terminus-attached glucose significantly enhances formation of the dimer subunit. These first two conclusions suggest that the phosphate moiety alone is responsible for the observed 4.5-fold increase in the dimer–tetramer equilibrium dissociation constant. Thirdly, once the glucose 6-phosphate is attached, the equilibrium subunit distribution of dimer and tetramer becomes refractory to the binding of either O_2 or CO ligands. These results, taken together with the established linkage between subunit dimer–tetramer equilibrium and O_2 binding for human haemoglobin [1,8], are not consistent with the glucose 6-phosphate moiety competing for the same binding site as does 2,3-bisphosphoglycerate on this macromolecule [3,5,20].

It is also of interest that two independent studies supporting the tetramer stabilization effects of organic phosphate salts show response curves (plot of apparent K_d versus organic phosphate concentration) to be biphasic [22,23]. In those studies, using inositol hexakisphosphate as the organic phosphate salt, the apparent K_d

decreased initially as the inositol hexakisphosphate concentration was increased, and subsequently increased beyond its initial value as the organic salt concentration was further increased. This biphasic phenomenon has been attributed to charge interactions of the free-phosphate negative charges with other sites on haemoglobin, e.g. ionic-strength effects. If, on the other hand, an infinitely high concentration of negative charges located at the 2,3-bisphosphoglycerate–protein interaction site does in fact promote dimer formation by virtue of site interaction only, then my present data would be consistent with covalent attachment of glucose 6-phosphate representing a high concentration of negative charges at the 'bisphosphoglycerate-binding site' and thereby represent the 'high-concentration'-limiting effects of negative charges at that site. The glucose 6-phosphate–haemoglobin model used in this study and the pyridoxal phosphate–haemoglobin model used by Benesch *et al.* [24] provide two working models that can be examined in thermodynamic detail without the ionic-strength effects induced by free charge groups at other sites on the protein.

It is well established that protein subunit interactions play a central role in mediating biological function at the molecular level [25]. In addition, protein phosphorylations have been implicated as a requisite for the activation of a variety of functional proteins in mammalian cells [4]. Suitable working models for studying the effects of phosphorylations on protein subunit assembly have not been established. Therefore attachment of phosphate moieties to well-characterized proteins would provide relevant functional models for studying the effects of these post-translational modifications on the thermodynamics of protein subunit assembly.

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